

Effects of lycorine on HL-60 cells via arresting cell cycle and inducing apoptosis

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Abstract As a natural anti-cancer alkaloid extracted from Amaryllidaceae, lycorine shows various biological effects on tumor cells. The survival rate of HL-60 cells exposed to lycorine was decreased in a dose-dependent manner with 1 μ M as the 50% inhibitory concentration (IC₅₀), cell growth was slowed down by arresting cell cycle at G2/M phase, and cell regeneration potential was inhibited. HL-60 cells exhibited typical apoptotic morphological changes, apoptotic DNA “ladder” pattern, and sub-G1 peak in cell phase distribution, showing apoptosis of HL-60 cells. To further understand the apoptotic molecular mechanism of lycorine on HL-60 cells, caspase activity was tested by colorimetric assay, and the expression of Bcl-2 and Bax proteins was examined by Western blotting. The increase of caspase-8, -9, -3 activities demonstrated that caspase was a key mediator of apoptotic pathways induced by lycorine. Under-expression of Bcl-2 and increase of Bax:Bcl-2 ratio showed that Bcl-2 family proteins were involved in apoptosis. Our finding suggests that lycorine can suppress leukemia growth and reduce cell survival via arresting cell cycle and inducing apoptosis of tumor cells.

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Keywords: Lycorine; Leukemia; HL-60 cell line; Cell cycle; Apoptosis

1. Introduction

Tumors are the diseases with proliferation disorder and apoptosis obstacle. The inhibition of proliferation and induction of apoptosis are regulated by a network of signaling pathways and transcription factors, which are possible targets for a rational tumor therapy [1,2]. For instance,

some anti-cancer reagents cause cell death through interfering with the processes of cell cycle [3] and some others cause cell death by apoptosis [4,5], which plays an important role in the balance between cell replication and cell death. Leukemia, one of the most threatening hematological malignant cancers today, has been found very sensitive to anti-cancer chemotherapeutic reagents which either interfere with cell cycles or cause apoptosis [6]. This chemical-reagent-sensitive potential of leukemia intrigues scientists to look for more specific and effective chemical drugs against it. As a very valuable source for novel chemotherapeutic reagents, natural plant compounds exhibit effective anti-tumor activities with wide range of mechanisms. For example, artemisinin and its derivatives mainly arrest cell cycle in G1 phase [7], while paclitaxel was proved to possess anti-microtubule activity and to arrest cell cycle at G2/M phase transition [8]. Therefore, it becomes an important research target and has been the aim of new drug design so as to find novel anti-leukemia chemotherapeutic medicines from natural products [9].

Lycorine is a natural alkaloid extracted from Amaryllidaceae. It showed various anti-tumor and anti-inflammatory activities. The research results revealed that lycorine could inhibit protein synthesis in eukaryotic cells [10] and the acetylcholinesterase enzyme [11]. Lycorine was also found to inhibit protein synthesis and cell apoptosis of MM46 in the presence of calprotectin [12]. However, the exact mechanism of lycorine against leukemia remains unknown. Here, we use human acute promyelocytic leukemia (APL) cell line HL-60, which is widely used as a leukemia cell model to study the cellular and molecular events, to investigate the molecular mechanism of lycorine against leukemia. In this study, we show that lycorine can suppress leukemia cell growth and reduce cell survival via arresting cell cycle and inducing apoptosis of tumor cells, which provides a new insight of lycorine serving as an anti-leukemia reagent.

2. Materials and methods

2.1. Cell culture and drugs

Human leukemia cell line HL-60 was purchased from American Type Culture Collection, and was cultivated in RPMI 1640 medium (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Invitrogen), 100 kU/L benzylpenicillin, and 100 mg/L streptomycin at 37 °C in a humidified atmosphere with 5% CO₂. Cells were diluted at a ratio of 1:5 every 2–3 days. Lycorine (Latoxan, France) was dissolved at 0.03 M in dimethyl sulfoxide (DMSO; Sigma) as a

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Abbreviations: IC₅₀, 50% inhibitory concentration; APL, acute promyelocytic leukemia; DMSO, dimethyl sulfoxide; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide; PBS, phosphate-buffered saline; AO, acridine orange; EB, ethidium bromide; PI, propidium iodide; IETD-pNA, Ile-Glu-Thr-Asp conjugated to *p*-nitroanilide; LEHD-pNA, Leu-Glu-His-Asp conjugated to *p*-nitroanilide; DEVD-pNA, Asp-Glu-Val-Asp conjugated to *p*-nitroanilide

stock solution and diluted in serum-free RPMI 1640 medium just before use. The maximum final concentration of DMSO in medium was smaller than 0.02% (v/v).

2.2. Cell survival rate and cytotoxicity assay (MTT)

Cell survival rate and cytotoxicity were measured with 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide (MTT) assay as described previously [13] with slight modification. Briefly, exponentially growing HL-60 cells were seeded at 1×10^4 cells/well in a 96-well tissue culture plate (BioCoat) with a total volume of 200 μ l per well. The lycorine concentrations ranging from 0 to 5 μ M were added immediately. After cells were incubated for 24 h, the relative cell viability was measured by scanning with an ELISA reader with a 570 nm filter and the 50% inhibitory concentration (IC₅₀) of lycorine on cells was calculated by MTT assay.

2.3. Cell counting

In order to examine the anti-proliferative effect of lycorine, growth curves were protracted by a manual count method. Cells treated with lycorine (1.25 μ M) or without lycorine were seeded at 1×10^4 cells/well in a 24-well tissue culture plate (BioCoat). After appropriate culture, viable cells were counted up to 5 days continuously by the trypan blue dye exclusion method.

2.4. Semisolid colony assay

HL-60 cells in the exponential growth phase were plated at 1.5×10^3 cells/well in a 24-well plate containing 2.5% methylcellulose, 10% FBS, RPMI 1640 medium and lycorine at different concentrations. After incubation for 7–10 days, colonies more than 50 cells were counted under an inverted microscope.

2.5. Fluorescence morphological examination

Cellular apoptotic morphological changes were studied by fluorescence microscope. Cells were harvested and washed three times with phosphate-buffered saline (PBS) after being incubated with different lycorine concentrations for 24 h, stained with 100 μ g/ml acridine orange and ethidium bromide (AO/EB; Sigma) for 5 min. Then, the types of cells were observed according to the color and structure [14] under a fluorescence microscope (Olympus).

2.6. DNA fragmentation assay

DNA fragmentation was analyzed after the extraction of low-molecular-weight DNA from cells, and 5×10^6 cells incubated with different lycorine concentrations or without lycorine for 24 h were harvested. Total DNA was extracted according to the procedure of Sellins et al. [15]. The DNA was separated on a 1.5% agarose gel and visualized under UV light by EB staining.

2.7. Flow cytometry

Flow cytometry was used to detect quantitatively apoptotic rate and the distribution of cell cycle. After being cultivated with medium alone or medium containing lycorine (5 μ M) at the indicated time (0, 12, 24, and 36 h), 1×10^6 cells were harvested and washed with PBS. Then, they were fixed with ice-cold 70% ethanol at 4 °C, stained with 50 mg/ml propidium iodide (PI; Sigma), and dissolved with 100 mg/L RNase A (Sigma). The sub-G1 peak (DNA content less than 2 N) and cell cycle distribution were measured with FAC-Scan Flow Cytometry (Becton–Dickinson) and analyzed by Cell Quest software.

2.8. Caspase activity assay

The cleavage activity of Ile-Glu-Thr-Asp conjugated to *p*-nitroanilide (IETD-pNA), Leu-Glu-His-Asp conjugated to *p*-nitroanilide (LEHD-pNA), and Asp-Glu-Val-Asp conjugated to *p*-nitroanilide (DEVD-pNA) was measured by using the FLICE/caspase-8, Mch6/caspase-9, and CPP32/caspase-3 colorimetric assay kits (BioVision), respectively. The recommended protocols were followed. Briefly, 2×10^6 exponentially growing HL-60 cells in 5 ml were treated with lycorine (5 μ M) for 0, 6, 12, 18, and 24 h at 37 °C. At the indicated time points, cells were pelleted by centrifugation and resuspended in 50 μ l chilled cell lysis buffer. The protein concentration was measured by using a micro BCA kit (Pierce). Then, we diluted 100 μ g protein

to 50 μ l cell lysis buffer for each assay and 50 μ l of 2 \times reaction buffer supplemented with 10 mM DTT was then added to each tube incubated at 4 °C. The substrates of IETD-pNA, LEHD-pNA, and DEVD-pNA were added into the tubes (5 μ l, 50 μ M), respectively. The formations of *p*-nitroanilide were measured by ELISA Microplate Reader (Bio-tek) at 405 nm before the samples were incubated for 1.5 h at 37 °C. Percent increase in caspases activities was determined by comparing those results of the indicated time points with controls.

2.9. Western blotting

2×10^6 exponentially growing HL-60 cells treated with lycorine at the indicated concentrations for 24 h were pelleted by centrifugation, washed with PBS (3 ml) two times and resuspended in 100 μ l of lysis buffer containing 20 mM HEPES, pH 7.4, 100 mM NaCl, 1% Nonidet P-40, 2% SDS, 1 mM EDTA, 1 mM EGTA, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 10 mg/ml leupeptin, and 1 mg/ml aprotinin, and sonicated. Protein concentration in supernatant was measured by using a micro BCA kit (Pierce). Protein (100 μ g) was electrophoresed for 2 h on 12% SDS-polyacrylamide gels and then transferred to polyvinylidene difluoride membranes (Millipore) by using an electroblotter for 12 h at 4 °C. In all cases, antibodies (Santa Cruz Biotech) raised against Bcl-2 or Bax (1:200) or α -tubulin (1:4000) were diluted in PBST containing 5% non-fat milk and membranes were incubated for 2 h with gentle agitation. The blots were washed for three times (10 min each) with PBST and incubated with a sheep anti-mouse conjugated to horseradish peroxidase (Santa Cruz Biotech, 1:2000 dilution in PBST containing 5% non-fat milk) for 2 h. After three successive washes (10 min each) with PBST, Western blotting chemiluminescence (ECL) reagent (Pierce) was used for

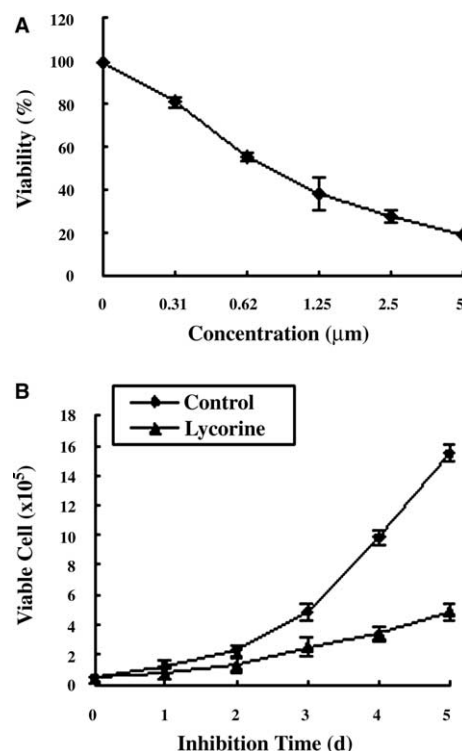


Fig. 1. Lycorine decreases the survival rate of HL-60 cells and slows down the cells' growth. (A) Effect of lycorine on the survival rate of HL-60. Cells were cultivated in RPMI 1640 medium with indicated concentrations of lycorine for 24 h. The cell survival rate was calculated by MTT assay. (B) Effect of lycorine on the growth curve of HL-60. HL-60 cells were treated by 1.25 μ M lycorine for indicated days. The cell proliferation was estimated by trypan blue dye exclusion method. Each value represents mean \pm S.D. of three independent experiments.

detection. The optical density of the immunoradiograms was quantified by densitometric scanning and data were analyzed by Sigma Plot.

2.10. Statistical analysis

The statistical difference between the groups was determined by AVOVA and Tukey's Studentized Range test. The level of $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Lycorine decreased the survival rate

MTT assays suggested that lycorine had some selective anti-tumor effects, the survival rate of HL-60 cells exposed to lycorine (0–5 μM) for 24 h was reduced in a dose-dependent manner, and the viability of cells was decreased from 100% to 18.95% (Fig. 1A). The concentration required to inhibit HL-60 cells growth by 50% (IC₅₀) was approximately 1 μM . Because lycorine was shown as a moderate toxic

Table 1
Colony formation of HL-60 cells after lycorine treatment

Lycorine (μM)	No. of colonies (per 1.5×10^3 cells)
0	179 ± 27
0.31	$96 \pm 17^*$
0.63	$25 \pm 9^*$
1.25	$3 \pm 2^*$
2.5	0^*

HL-60 cells in the exponential growth phase were plated in a 24-well plate with indicated concentrations of lycorine. After incubation for 7–10 days, colonies with more than 50 cells were counted under an inverted microscope. Values are means \pm S.D. in triplicate cultures (asterisk symbol indicates $P < 0.05$ (*) compared with the control group).

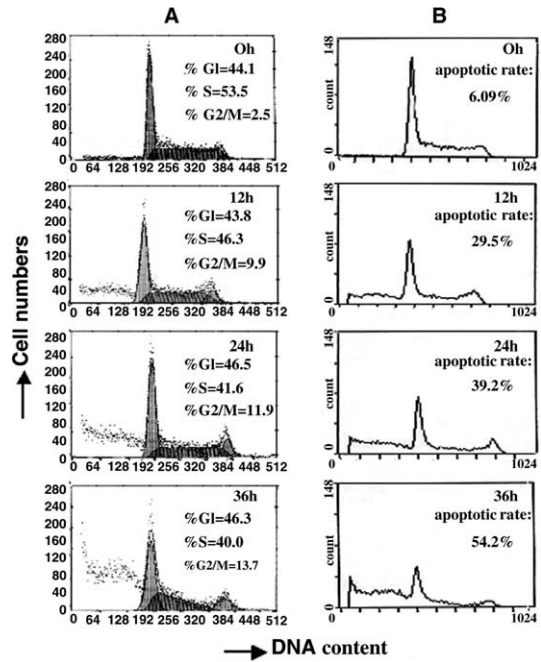


Fig. 2. Lycorine increases the proportion of G2/M phase cells and apoptotic rates of HL-60 cells. After being treated with lycorine (5 μM) or without for indicated times, cells were harvested and washed with PBS, fixed with ice-cold 70% ethanol, stained with PI, and treated with RNase A. DNA content and cell cycle distribution were measured by flow cytometry and analyzed by Cell Quest Software. (A) HL-60 cells cultivated with lycorine (5 μM) exhibit a decrease in S phase cells corresponding to an increase in G2/M phase cells. The percentage of G2/M phase cells was increased gradually from 2.5% to 13.7% when the induction time was persisted from 0 to 36 h. (B) 5 μM lycorine causes a significant increase of apoptotic rate of HL-60 cells from 6.09% to 54.2% in a time-dependent manner.

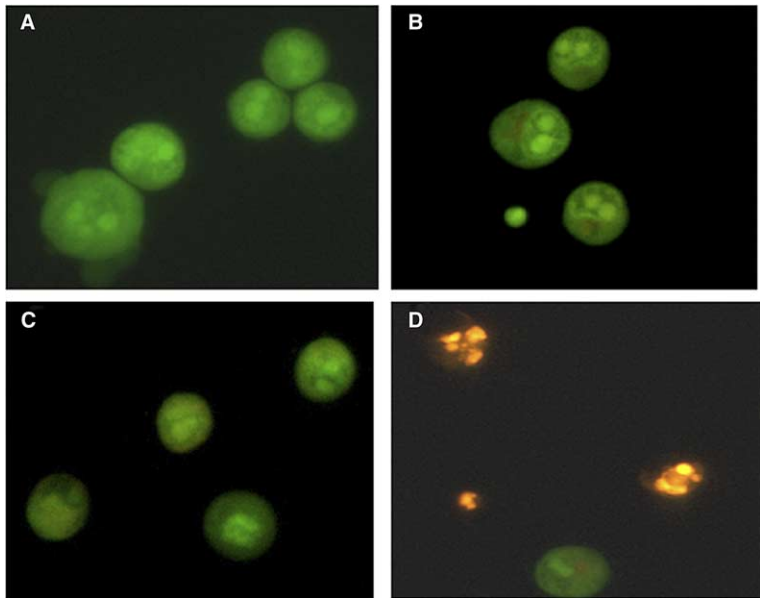


Fig. 3. Lycorine induces apoptotic morphological changes on HL-60 cells. After being treated with indicated concentrations of lycorine for 24 h, HL-60 cells were harvested, washed with PBS, and stained with AO/EB (100 $\mu\text{g}/\text{ml}$). Cell morphology was observed under fluorescence microscopy. Green live cells show normal morphology in control (A); green early apoptotic cells show nuclear margination and chromatin condensation with 1.25 μM (B) or 2.5 μM (C) lycorine treatment. Orange later apoptotic cells show fragmented chromatin and apoptotic bodies after 5 μM lycorine treatment (D) ($\times 400$).

drug, we examined the effect of lycorine on the survival rate of other tumor and non-tumor cell lines, such as human multiple myeloma cell line ARH-77, mouse fibroblast cell line NIH3T3, and human recombinant cell line HEK293. We found that lycorine at the same concentration could decrease the survival rate of tumor cell lines, HL-60 and ARH-77, more than that of non-tumor cell lines, NIH3T3 and HEK293, and HL-60 cell line was the most sensitive one to lycorine (data not shown).

3.2. Anti-proliferation and cell cycle arrest of lycorine

Cell proliferation was significantly inhibited by lycorine (1.25 μ M), examined by trypan blue dye exclusion method (Fig. 1B). The density of lycorine-treated HL-60 cells decreased to one-third of that of the control after five days' treatment. In the semisolid culture, the number of colonies decreased significantly in a dose-dependent manner in experimental groups (Table 1), suggesting that lycorine effectively reduced the malignancy and suppressed the regeneration potential of tumor cells. Since HL-60 cell line was highly sensitive to the anti-proliferation effect of lycorine, we examined whether lycorine could interfere with cell cycles using the flow cytometry. We found that after HL-60 cells were treated with 5 μ M of lycorine, the percentage of cells at S phase decreased, corresponding to an increase in the percentage of cells at G2/M phase (from 2.5% to 13.7%) (Fig. 2A), indicating an arrest of cell cycle at G2/M phase by lycorine.

3.3. Occurrence of apoptosis induced by lycorine

We examined that lycorine induced apoptosis of HL-60 cells through using the fluorescence microscope after staining cells with AO/EB. Green live cells with normal morphology were observed in control group (Fig. 3A). Green early apoptotic cells with nuclear margination and chromatin condensation were observed after lycorine treatment at concentrations of 1.25 and 2.5 μ M (Fig. 3B and C), while orange later apoptotic cells with fragmented chromatin and apoptotic bodies were seen after the treatment of lycorine at concentration of 5 μ M (Fig. 3D).

No DNA fragmentation was observed when lycorine was applied at concentration of 1.25 μ M, but when the lycorine concentration was increased to 2.5 or 5 μ M, an apoptotic DNA "ladder" pattern was obviously observed (Fig. 4A). Apoptotic rates increased from 6.09% to 54.2% in a time-dependent manner after lycorine (5 μ M) induction when cells were tested by the flow cytometry (Fig. 2B).

3.4. Caspase activation

A remarkable activation of caspase-8, caspase-9, and caspase-3 was recorded in lysates from cells treated with 5 μ M lycorine at different time points (Fig. 4B), and caspase activation persisted for more than 24 h. The activation of caspase-3 was increased most significantly.

3.5. Under-expression of Bcl-2 protein and increase in Bax:Bcl-2 ratio by lycorine

The effect of lycorine on the expression of Bcl-2 and Bax proteins was determined by immunoblot. α -tubulin was used for normalization and verification of protein loading. After being normalized to α -tubulin, the expression of Bcl-2 was decreased significantly in a dose-dependent manner, while Bax

protein level was increased slightly (Fig. 5A and B). The ratio of Bax:Bcl-2 was dramatically increased in a dose-dependent manner (Fig. 5C).

4. Discussion

Cancer and many human diseases are cell cycle diseases. Cell cycle coordination takes place mainly at G1/S and G2/M phase transitions by a series of checkpoints. It has been shown that

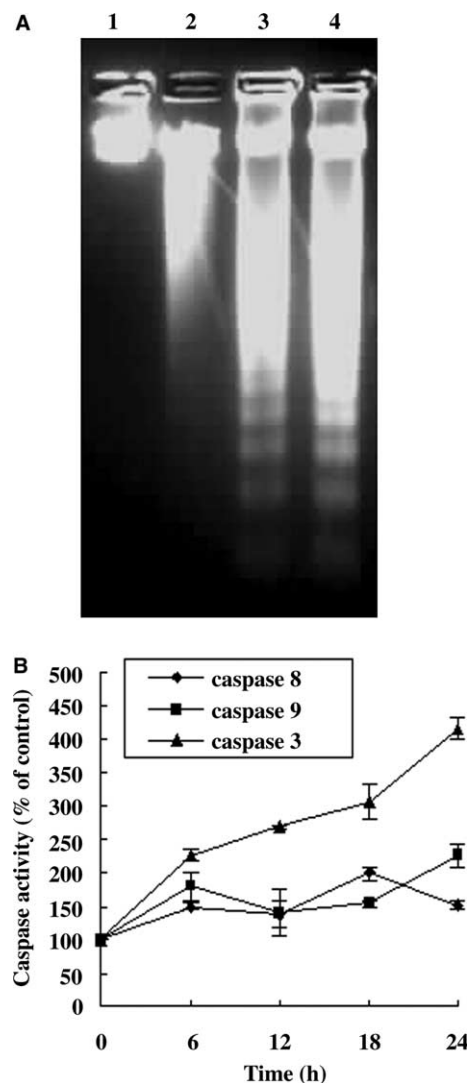


Fig. 4. Lycorine induces apoptosis on HL-60 cells via activating caspases pathways. (A) Inducing DNA fragmentation on HL-60 cells by lycorine. After lycorine treatment, the cellular DNA was extracted, dialyzed and separated on a 1.5% agarose gel and visualized under UV light by EB staining. Lane 1: control; lanes 2–4: lycorine (1.25, 2.5, and 5 μ M for 24 h, respectively). (B) Activation of caspase-8, caspase-9, and caspase-3 by lycorine. HL-60 cells were incubated with lycorine (5 μ M) for indicated time before the caspase-8 substrate IETD-pNA, or caspase-9 substrate LEHD-pNA, or caspase-3 substrate DEVD-pNA (50 mM) was added. Assay mixtures were incubated for 1.5 h at 37 °C followed by measurement of the absorbance at 405 nm. Percent increase in caspases activities was determined by comparing those results at the indicated time points with the level of uninduction. Results are presented as means \pm S.D. (three independent experiments).

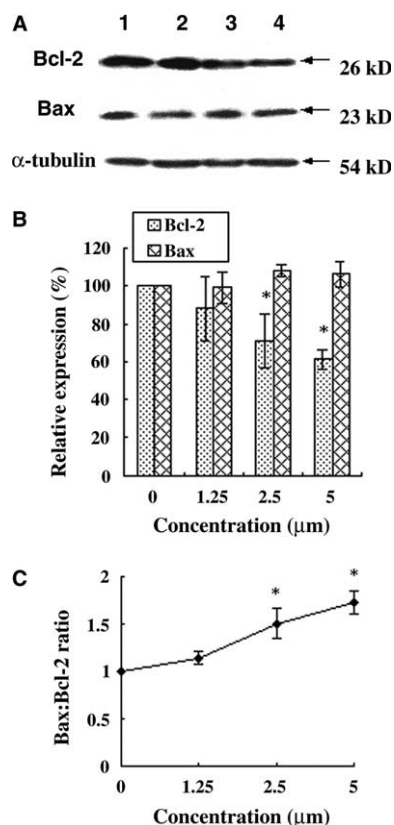


Fig. 5. Effect of lycorine on expression of Bcl-2 and Bax in HL-60 cells. Exponentially growing HL-60 cells were treated with indicated concentrations of lycorine for 24 h, cell lysates were prepared and protein level of Bcl-2 and Bax was determined by immunoblot analysis. (A) The expression of Bcl-2 and Bax after lycorine induction. α -tubulin was used for normalization and verification of protein loading. Lane 1: control; lanes 2–4: HL-60 cells were treated with 1.25, 2.5, or 5 μ M lycorine, respectively. (B) Quantitative Bcl-2 and Bax expression after normalization to α -tubulin ($n = 2$ at each concentration point). The expression of Bcl-2 was decreased significantly, while Bax protein level was increased slightly after lycorine induction. (C) The Bax:Bcl-2 ratio corresponding to indicated concentrations of lycorine. Compared to control, the ratio of Bax:Bcl-2 was increased significantly when 2.5 and 5.0 μ M lycorine were applied. Values are means \pm S.D. (asterisk symbol indicates $P < 0.05$ (*) compared with the control group).

the activities of many regulatory factors of checkpoints are lost or arrested during the process of tumorigenesis [16], and found that some anti-tumor reagents could restore the altered regulatory checkpoints [17]. Many anti-tumor reagents can arrest cell cycle and then induce cell apoptosis [18,19]. Cell cycle arrest may ensure that cells have time to repair the damages [20,21], whereas apoptosis may eliminate the damaged cells [22]. Our study demonstrated that lycorine had anti-tumor effect by arresting G2/M HL-60 cells and inducing cell apoptosis.

The mechanisms of apoptosis involve in mainly two signaling pathways, including mitochondrial pathway and cell death receptor pathway. The key element in mitochondrial pathway is the efflux of cytochrome *c* from mitochondria to cytosol, where it subsequently forms a complex (apoptosome) with Apaf-1 and caspase-9, leading to the activation of the caspase-3 [23]. The cell death receptor pathway is characterized by binding cell death ligands and cell death receptors, and subsequently activates caspase-8 and caspase-3 [24,25]. It can be

seen that caspases are always at the center of apoptotic machinery. Several caspases are shown to be key executors of apoptosis mediated by various anti-tumor drugs [26]. Our study indicated that mitochondrial pathway and death receptor signaling pathway were both involved in apoptosis induced by lycorine.

The Bcl-2 family proteins that consist of anti-apoptotic and pro-apoptotic members determine life-or-death of a cell [27]. Bcl-2 oncoprotein located on the outer mitochondrial membrane is important for the suppression of apoptosis [28]. Bcl-2 can stabilize the mitochondria permeability transition and avoid the release of cytochrome *c* to prevent caspase activation [29]. Upregulation of Bax together with the downregulation of Bcl-2 and increase in the ratio of Bax:Bcl-2 could be seen during apoptotic death [30]. Our findings suggest that these proteins are involved in the intracellular pathway leading to apoptotic death induced by lycorine. Furthermore, the down-regulation of Bcl-2 would inevitably promote the activation of caspase.

In conclusion, lycorine served as anti-cancer function, which could arrest cell cycle at G2/M phase and induce apoptosis of HL-60 cells. We further proved that apoptosis of HL-60 cells was induced by decreasing the level of Bcl-2, increasing the ratio of Bax:Bcl-2, and increasing the activation of caspase-8, caspase-9, and caspase-3. Thus, our findings provide new insights into the molecular mechanism of the anti-leukemia activity of lycorine.

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